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Redox regulation of glucitol dehydrogenase

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Abbreviations

Fru, fructose; Glc, glucose; Gol, glucitol; GolDHase, glucitol dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; *Ppe*, *Prunus persica*; TRX, thioredoxin

Abstract

Glucitol (Gol) is a major photosynthetic product in plants from the Rosaceae family. Herein we report the molecular cloning, heterologous expression, and characterization of Gol dehydrogenase (GolDHase, EC 1.1.1.14) from peach (*Prunus persica*) fruits. The recombinant enzyme showed kinetic parameters similar to those reported for orthologous enzymes purified from apple and pear fruits. The activity of recombinant GolDHase was strongly inhibited by Cu^{2+} and Hg^{2+}, suggesting that it might have cysteine residues critical for functionality. Oxidizing compounds (like diamide, hydrogen peroxide, and oxidized glutathione) inactivated the enzyme, whereas its activity was restored after incubation with reduced glutathione and thioredoxin from *Escherichia coli*. Recombinant thioredoxin *h* from peach fruits also recovered the activity of oxidized GolDHase. Our results suggest that peach fruit GolDHase could be redox regulated *in vivo* and this would be of relevance to determine carbon assimilation and partitioning in plants accumulating sugar-alcohols.

Key words:

Glucitol dehydrogenase, glucitol metabolism, peach, *Prunus persica*, redox regulation, thioredoxin

Introduction

Carbon partitioning is critical for plant growth and productivity. For this reason, many efforts have been done to understand starch and sucrose metabolism, the major photosynthetic products in plants (Iglesias and Podestá 2005; Stitt et al. 2010). In most species, sucrose is the main molecule used for carbon partitioning between source and sink tissues. In addition to starch and sucrose, plants from the Rosaceae family utilize a reduced carbohydrate (sugar-alcohol) to transport carbon between tissues, constituting an alternative to the canonical route. Glucitol (Gol), also known as sorbitol, is a metabolite produced in the cytosol of photosynthetically active leaves of many rosaceous plants, such as apple, pear, peach, and plum (Figueroa et al. 2010; Loescher and Everard 2000). Genome projects on rosaceous plants revealed that diploid representative species of this family have very small, compact genomes: 200 Mb for woodland strawberry (*Fragaria vesca*) (Shulaev et al. 2011) and 227 Mb in the case of peach (*Prunus persica*) (Verde et al. 2013). These plants exhibit a significant diversity in growth (ranging from herbaceous to tree forms) and represent a suitable system for comparative studies on genome evolution.

Gol synthesis and accumulation are restricted to the subfamily Spiraeoideae (which includes apple and peach, among other important rosaceous crops); while in Rosoideae and Dryoideae this polyol is absent. Genomic data suggest that massive biosynthesis and accumulation of the polyol in these species are, in part, linked to expansion in the number of genes in particular families; as those coding for NADP-dependent aldose-6-phosphate reductase (EC 1.1.1.200), NAD-dependent Gol dehydrogenase (EC 1.1.1.14, GolDHase), and Gol transporters (Verde et al. 2013). Aldose-6P reductase catalyzes the reduction of glucose-6P (Glc-6P) to Gol-6P (Figueroa and Iglesias 2010; Hirai 1981; Kanayama and Yamaki 1993; Negm and Loescher 1981; Zhou et al. 2003b), being the phosphate group subsequently hydrolyzed by a specific phosphatase (EC 3.1.1.50) (Zhou et al. 2003a). Gol is then exported to heterotrophic tissues, where it is converted into fructose (Fru) in a reaction catalyzed by GolDHase (Lo Bianco and Rieger 2002a, b; Negm and Loescher 1979; Nosarszewski et al. 2004; Oura et al. 2000): Gol + NAD^+ ⇆ Fru + NADH + H^+.

GolDHase belongs to the ubiquitous dehydrogenase/reductase superfamily (Jörnvall 2008; Persson et al. 2008) and is mostly located in the cytosol of non-photosynthetic cells (Loescher and

Everard 2000). Analysis by immunogold electron microscopy demonstrated that in apple the enzyme is also present in fruit plastids and leaf vacuoles, indicating that it might play a differential role for Gol metabolism in different tissues (Wang et al. 2009). GolDHase is highly expressed in heterotrophic tissues (young leaves and fruits) of plants synthesizing Gol (Bantog et al. 2000; Nosarszewski et al. 2004; Park et al. 2002; Yamada et al. 1999; Yamada et al. 2001; Yamada et al. 1998). The transcript of the At5g51970 gene (coding for a GolDHase) is expressed in multiple organs in the non-rosaceous plant *Arabidopsis thaliana*; and its disruption reduces the utilization of exogenous Gol and decreases osmotic stress tolerance in the mutants (Aguayo et al. 2013).

GolDHase from fruits of apple (Yamaguchi et al. 1994) and pear (Oura et al. 2000) have been purified and kinetically characterized, and the cDNAs encoding the enzyme in fruits were cloned from apple (Yamada et al. 1998), loquat (Bantog et al. 2000), peach (Yamada et al. 2001), and plum (Guo et al. 2012). Several cDNAs that code for different GolDHase isoforms in apple fruits were expressed in *Escherichia coli* cells, although the recombinant proteins were poorly characterized. Yamada et al. (1998) obtained the apple enzyme fused to thioredoxin (TRX) from *E. coli* (*Eco*TRX) and determined a K_m for Gol, considerably higher to that observed for the enzyme purified from the source (Yamaguchi et al. 1994), probably due to the presence of the fusion tag. Subsequently, Park et al. (2002) measured K_m values for Gol and Fru of the recombinant GolDHase isoform 3 (MdSDH3) from Fuji apple, which were similar to those previously reported for the enzyme purified from the same fruit (Yamaguchi et al. 1994). Wang et al. (2009) cloned and expressed two isoforms of apple GolDHase (MdSDH5 and MdSDH6) as GST fusion products and assayed their activity, but no kinetic parameters were determined. The recombinant enzymes from tomato (Ohta et al. 2005) and *A. thaliana* (Aguayo et al. 2013) were characterized in more detail; though, these studies contributed scarcely to the understanding of carbon partitioning related with sugar-alcohols accumulation, because Gol is not a major photosynthetic product in such plants. Recently, Guo et al. (2012) determined the kinetic parameters, optimal temperature and pH, and substrate specificity of a recombinant GolDHase from fruit plum (a rosaceous specie); although the enzyme regulatory properties were not analyzed.

In recent years, several works were performed to better understand the molecular mechanisms

involved in the regulation of carbon metabolism during fruit peach development (Lo Bianco and Rieger 2002b; Morandi et al. 2008) and storage (Borsani et al. 2009; Lara et al. 2009; Lara et al. 2011; Lauxmann et al. 2014). However, the information on kinetic, structural, and regulatory properties of enzymes from GolI metabolism remains limited. In this work, we developed a simple method for recombinantly obtain a peach fruit GolDHase (*Ppe*GolDHase) with a high level of purity. Also, we report the *in vitro* redox regulation of the enzyme by a recombinant TRX*h* (*Ppe*TRX*h*) and discuss its significance for the modulation of carbon metabolism in this species.

Results and Discussion

Phylogenetic analysis, expression, and purification of Ppe*GolDHase*

Several sequences of plant GolDHases have become available in the last decade. Genome projects for apple (Velasco et al. 2010) and peach (Verde et al. 2013) have revealed the existence of 17 and 6 genes coding for GolDHases, respectively; whereas most non-rosaceous species have only one or two of such genes. Therefore, it seems that the expansion of this gene family is closely related to the function of GolI as a photosynthetic product in plants from the Rosaceae family (Velasco et al. 2010; Verde et al. 2013). We constructed a phylogenetic tree of GolDHases (Figure 1) from sequences coding for the enzyme from apple, peach, and other plants of various families (described in Supplementary Table S1 and Supplementary Figure S1). Figure 1 shows that all the sequences of GolDHases found in *Malus* x *domestica* (apple, sequences 1-12) are in the same group. In a close branch we observed the enzymes from *Prunus* species, encoded by cDNAs cloned from fruits of peach (sequences 14 and 17) (Yamada et al. 2001), plum (sequence 18) (Guo et al. 2012), and cherry (sequence 19) (Gao et al., direct submission to the NCBI database, unpublished). It is worth noting that sequence 14 (reported in the peach genome project) (Verde et al. 2013) is identical to sequence 17, previously published by Yamada et al. (2001). The remaining sequences from peach are arranged in a different group that also includes those from other dicotyledonous plants (Figure 1). GolDHases from the rosaceous species *Fragaria vesca* (woodland strawberry, sequence 22) and *Fragaria* x *annannasa* (strawberry, sequence 28) are in a different group

(Figure 1). An interesting feature of this phylogenetic tree is that the sequences from monocotyledonous plants (family Poaceae) are within the same branch. A similar trend was observed for the enzyme from plants of the Brassicaceae and Fabaceae families (Figure 1). The most divergent sequences are those from cucumber (sequence 30), *Physcomitrella patens* (sequence 41), *Selaginella moellendorffii* (sequence 42), and the conifer Sitka spruce (sequence 43).

In this work, we cloned the gene coding for *Ppe*GolDHase (NCBI accession number AB025969) using RNA from peach fruit mesocarp as template. The amplified DNA comprises an open reading frame of 1,104 bp encoding a putative protein of 367 amino acids with theoretical pI of 6.46 and estimated mass of 39.1 kDa, identical to that described by Yamada et al. (2001) (sequences 14 and 17 in Figure 1, NCBI accession number BAA94084). By the time we were cloning this cDNA, the peach genome project was in an early stage and the only sequence available for the respective GolDHase was the one reported by Yamada et al. (2001). This gene was subcloned into the pET19b vector and the recombinant enzyme was expressed with a His-tag at the N-terminus, to obtain a protein of 390 amino acids and calculated molecular mass of 41.9 kDa. When transformed cells were grown in LB medium the recombinant protein was primarily found in the insoluble fraction and the soluble protein showed very low activity. Replacement of the culture medium by LB-Glc, YT2X, or TB did not improve the amount of active *Ppe*GolDHase recovered in the soluble fraction (Figure 2).

It has been reported that GolDHases harbor a Zn^{2+} ion necessary for catalytic activity (Banfield et al. 2001; Pauly et al. 2003) and the plant enzyme contains a second Zn^{2+} ion that is critical for maintaining the protein's structure (Yamada et al. 2001). Considering this, we expressed *Ppe*GolDHase in LB media supplemented with different concentrations of $ZnCl_2$ to find an effective procedure to significantly improve GolDHase activity in the soluble fraction. Based on results shown in Figure 2, we chose LB supplemented with 0.1 mM $ZnCl_2$ (which optimally increased the activity by ~14-fold) to grow *E. coli* cells producing the recombinant *Ppe*GolDHase. Although such expression condition rendered high protein contents in the insoluble fraction (Figure 3, lane 1), purification of the soluble extract (Figure 3, lane 2) provided a highly pure (>95%) protein with a molecular mass of ~42 kDa (Figure 3, lane 3) having activity of GolDHase (specific activity of 16 U mg^{-1}). Gel filtration chromatography on Superdex 200 indicated that the purified

*Ppe*GolDHase has a molecular mass of 160 kDa (data not shown), in agreement with the tetrameric structure determined for this enzyme from Japanese pear (Oura et al. 2000) and plum (Guo et al. 2012) fruits.

Kinetic characterization of Ppe*GolDHase*

We determined the optimal pH values for *Ppe*GolDHase in both directions of catalysis (Figure 4). In the physiological direction (oxidation of Gol) the highest activity was observed at pH 10.0; whereas in the opposite direction (reduction of Fru) the activity showed a peak at pH 6.0. As shown in Figure 4, slightly lower activity values were observed when assays were performed in buffers CAPS or MES in comparison with Bis-Tris Propane. These results are similar to those observed for GolDHases from tomato (Ohta et al. 2005), Japanese pear (Oura et al. 2000), maize (Doehlert 1987), and plum (Guo et al. 2012). The enzyme remained fully active after incubation at different pH values (from 5.0 to 11.0) for 10 min at room temperature (assayed under standard conditions, data not shown).

To further characterize *Ppe*GolDHase we determined the kinetic parameters for the substrates in both directions of catalysis at pH values where it reached the highest activity (Table 1). We found that the V_{max} and $S_{0.5}$ values for Gol and Fru are considerably dissimilar among different plant GolDHases (Table 1 and Supplementary Table S2). However, in all the plant enzymes characterized so far, including *Ppe*GolDHase, the catalytic efficiency for Gol oxidation is higher compared with that for Fru reduction (Table 1 and Supplementary Table S2). Therefore, the catalytic conversion of Gol into Fru would be favored even in plants where Gol is not a primary photosynthetic product (like maize and tomato). A detailed comparison between the kinetic parameters found for *Ppe*GolDHase and those corresponding to the enzyme from other rosaceous plants shows that: (i) the $S_{0.5}$ for Gol is similar to that of apple GolDHase and 2-fold lower than those reported for the enzymes from pear and plum; (ii) the $S_{0.5}$ values for NAD^+ and NADH are 4- and 2-fold higher compared to those determined for the plum enzyme; and (iii) the $S_{0.5}$ for Fru is 2-, 9-, and 30-fold lower than those respectively obtained for the enzymes from apple, plum and pear (compare data in Table 1 and Table S2). Irrespective of the plant source, the apparent

affinity of GolDHases toward Fru is considerably low. It has been informed that only the keto tautomer of Fru binds to the enzyme at the time of hydride delivery from NADH (Pauly et al. 2003) and it is worth noting that this tautomer represents less than 1% of the total (Barclay et al. 2012), which could explain the high $S_{0.5}$ for Fru determined in all plant GolDHases characterized so far (Table 1 and Supplementary Table S2).

Additionally, we tested the effect of different metabolites on *Ppe*GDHase activity in the physiological direction of the reaction. No significant differences were observed when Glc-1P, Glc-6P, Glc-1,6-bisP, Fru-6P, Fru-1,6-bisP, Fru-2,6-bisP, Gol-6P, mannose-6P, trehalose, phosphoenolpyruvate, pyruvate, ribose-5P, ATP, ADP, AMP, ADP-Glc, UTP, UDP-Glc, or Pi (10 mM each) were added to the enzyme assay mixture (data not shown). These results suggest that *Ppe*GolDHase would not be regulated by metabolites. Conversely, the enzyme was strongly inhibited by Cu^{2+} and Hg^{2+} with $I_{0.5}$ values in the nM range (Table 2), as it was previously reported for other plant GolDHases (Negm and Loescher 1979; Oura et al. 2000). The $I_{0.5}$ values determined for Ni^{2+}, Zn^{2+}, and Co^{2+} were three to four orders of magnitude higher than those for Cu^{2+} and Hg^{2+} (Table 2), whereas the activity of the enzyme did not change significantly in presence of 5 mM Mg^{2+}, Ca^{2+}, or Mn^{2+} (data not shown).

Oxidizing and reducing agents modify the activity of Ppe*GolDHase*

A common mechanism affecting enzyme activity *in vivo* is the oxidation of Cys residues mainly by (but not limited to) reactive oxygen species, reactive nitrogen species, and oxidized glutathione (GSSG). Analysis of the amino acid sequence of *Ppe*GolDHase shows that it has 13 Cys residues (Supplementary Figure S1) and it is tempting to speculate that some of them are part of a redox center. The inactivation caused by Cu^{2+} and Hg^{2+} suggests that there might be important Cys residues for the enzyme activity. To analyze this issue, *Ppe*GolDHase was incubated with increasing concentrations of several oxidizing compounds and the alkylating agent iodoacetic acid (IAA). To assess the respective modifying strength, the interaction of each compound with the enzyme was kinetically analyzed as described in Appendix S1. Results indicated that oxidation (also alkylation) by different agents causes enzyme inactivation in a time

and concentration dependent manner (as exemplified for the effect of H_2O_2 in Supplementary Figure S2). Using the model developed by Kitz and Wilson (1962) for the irreversible inhibition of enzymes we estimated the kinetic parameters shown in Table 3. Similar *k"* values were obtained with the non-physiological agents (diamide and IAA), and the inactivating capacity of H_2O_2 was in the same order of magnitude. The latter compound showed to be the more effective between the physiological oxidants tested, as it inactivated the enzyme with a *k"* one or two orders of magnitude higher than sodium nitroprusside (SNP, which generates nitric oxide and other reactive nitrogen species) (Piattoni et al. 2013) or GSSG, respectively. Results agree with the idea that *Ppe*GolDHase might be inactivated *in vivo* under oxidizing conditions.

To further investigate the probable occurrence of a physiological redox regulation process, the reversibility of the oxidation was analyzed by using the biological agents TRX and reduced glutathione (GSH). As shown in Figure 5, oxidized *Ppe*GolDHase (~5% residual activity) recovered the activity (up to ~1.2-fold of the initial value) after 60 min incubation with 50 µM *Eco*TRX. Similarly, treatment with 10 mM GSH completely rescued *Ppe*GolDHase from its inactive state, up to ~2.0-fold of the initial activity (Figure 5). The effect of GSH could be of relevance *in vivo*, as its intracellular concentration in plant tissues is 2-5 mM (Foyer and Noctor 2005) and it was found effective to recover the activity of oxidized glyceraldehyde-3P dehydrogenase from *A. thaliana* (Holtgrefe et al. 2008) and wheat leaves (Piattoni et al. 2013). These results prompted us to analyze the ability of TRX*h* from peach fruit (*Ppe*TRX*h*) to act as an effective reducing agent on *Ppe*GolDHase. For detailed information on the phylogenetic analysis, cloning, expression, purification, and characterization of *Ppe*TRX*h* see Supplementary Appendix S2 and Supplementary Figures S3-S6. We calculated the midpoint reduction potential (E_m) for *Ppe*GolDHase and *Ppe*TRX*h* to determine if both proteins could participate in a redox cascade (Figure 6). GolDHase activity and TRX*h* fluorescence were measured at varying reduction potentials (ranging from -0.378 to -0.023 V) using the pair 2ME-HEDS as redox buffer. Values of E_m thus calculated were -0.295 V for *Ppe*GolDHase and -0.263 V for *Ppe*TRX*h* (see Figure 6), which are comparable to those reported for many redox-regulated enzymes, including TRX, ferredoxin-TRX reductase, and several TRX-activated plant enzymes (Hicks et al. 2007; Hirasawa et al. 1999; Hutchison et al. 2000; Hutchison and Ort 1995). The

proximity between the *Ppe*TRX*h* and *Ppe*GolDHase E_m values agrees with a model where these proteins might be part of a redox cascade.

Thereafter, we analyzed if *Ppe*TRX*h* (in its reduced form) was able to affect the redox state of *Ppe*GolDHase. The enzyme was first incubated with 0.2 mM H_2O_2 at room temperature, which lowered its initial activity by 28% (5 min incubation) and 64% (10 min incubation). Figure 7 shows how different concentrations of reduced *Ppe*TRX*h* could restore the activity of the enzyme with a dependence on the degree of the primary oxidative treatment. Thus, incubation with 50 µM *Ppe*TRX*h* increased the activity of *Ppe*GolDHase that had been oxidized for 10 or 5 min to, respectively, a value close to the initial (18.2 U mg^{-1}) or even 2-fold higher than the initial (the non-oxidized enzyme). In fact, an enhancement in the activity of up to ~2.5-fold was observed for the non-oxidized enzyme after the incubation of with *Ppe*TRX*h* (Figure 7). We hypothesize that critical Cys residues in the enzyme could be oxidized to different degrees (i.e. to disulfide, sulfinic acid or sulfonic acids) by H_2O_2, which distinctively limit the ability of *Ppe*TRX*h* as a redox agent (Piattoni et al. 2013). In association, another feasible explanation for this phenomenon is that oxidation of any of the Cys residues involved in Zn^{2+} binding (Yamada et al. 2001) might lead to metal release causing *Ppe*GolDHase inactivation by altering the catalytic site and/or the structure of the protein (Giles et al. 2003). Regardless, the concentration of *Ppe*TRX*h* necessary to restore *Ppe*GolDHase activity is similar to the values reported for the regulation by TRX of chloroplast Fru-1,6-bisphosphatase from spinach leaves (Mora-García et al. 1996), potato tuber ADP-Glc pyrophosphorylase (Ballicora et al. 2000), phosphoenolpyruvate carboxylase protein kinase from maize leaves (Saze et al. 2001), and cytosolic glyceraldehyde-3P dehydrogenases from wheat leaves (Piattoni et al. 2013).

Figure 7 also illustrates that recombinant *Ppe*GolDHase is not fully active and has a partially oxidized stable conformation. The higher activity level (30 U mg^{-1}) was also obtained by incubating the enzyme with 20 mM GSH for 20 min (data not shown). Considering that the kinetically characterized *Ppe*GolDHase (Table 1) was in a partially oxidized state, the kinetic constants were re-evaluated and determined for the enzyme pre-treated with 20 µM *Ppe*TRX*h*. Under this condition, the V_{max} increased by 2-fold in both directions of the reaction when compared to data in Table 1, which agrees with results presented in Figure 7. Conversely, no significant changes were observed in the $S_{0.5}$ for substrates.

Overall, these observations strengthen the hypothesis that activity of *Ppe*GolDHase could be *in vivo* modulated by *Ppe*TRX*h*.

Concluding remarks

Carbon partitioning in plants producing sugar alcohols is not well understood and few studies deal with the characterization of enzymes regulating this pathway. In this paper, we report a simple and efficient method for obtaining, with high purity, considerable amounts of pure GolDHase and TRX*h* from peach fruits, suitable for structural and kinetic studies. Recombinant *Ppe*GolDHase showed similar properties to those reported for the homologous enzyme from different sources, including apple (Negm and Loescher 1979; Yamaguchi et al. 1994), pear (Oura et al. 2000), plum (Guo et al. 2012) and tomato (Ohta et al. 2005). As it was previously described for other GolDHases from plants (Negm and Loescher 1979; Oura et al. 2000; Yamaguchi et al. 1994), the recombinant enzyme characterized herein was strongly inactivated by divalent cations like Cu^{2+} and Hg^{2+}. This result suggests that there might be Cys residues that are critically involved in establishing the enzyme activity. Therefore, we evaluated the effect of different oxidizing agents on the activity of *Ppe*GolDHase. Interestingly, the recombinant enzyme was inactivated by different sulfhydryl oxidants and by the thiol alkylating agent IAA. The reversibility of the oxidation was tested by using the reducing agents GSH and *Eco*TRX, and with both compounds the activity of *Ppe*GolDHase was recovered. To analyze the effect of the TRX from the homologous plant species, that also shares the same intracellular localization, we cloned *Ppe*TRX*h*. Our results showed that the amount of *Ppe*TRX*h* necessary to recover the activity of the oxidized GolDHase is between 25 and 50 µM, values similar to those reported for other redox regulated plant enzymes (Ballicora et al. 2000; Mora-García et al. 1996; Piattoni et al. 2013; Saze et al. 2001). The E_m of the proteins also were within the range reported for other redox regulated enzymes (Hicks et al. 2007; Hirasawa et al. 1999; Hutchison et al. 2000; Hutchison and Ort 1995) and close enough to participate in a redox cascade.

Our results led us to propose a metabolic scenario (Figure 8) in which, under normal conditions, GolDHase could be fully active and Gol accumulated into heterotrophic tissues (fruits) would be converted

into Fru, with the concomitant production of NADH. Morandi et al. (2008) proposed that this pathway could be more beneficial than that catalyzed by sucrose synthase, as the generated NADH could be used for biosynthetic reactions and respiration, both important during fruit growth. Fru generated either by GolDHase or sucrose synthase could be used into different metabolic pathways or stored as starch within the plastid. However, under conditions producing an oxidizing environment, activity of GolDHase would decrease due to oxidation, thus leading to accumulation of Gol. Reversion of such a situation could depend on levels of GSH and reduced TRX, which is tied to an opposite situation of reduced intracellular environment. Several works proposed that sugar alcohols (including Gol) could act as radical scavengers, therefore protecting cell integrity under oxidizing conditions (Jennings and Burke 1990; Shen et al. 1997; Smirnoff 1993; Smirnoff and Cumbes 1989). Altogether, our results suggest that an oxidizing environment would trigger the inhibition of GolDHase, resulting in an increase in Gol levels, which itself would help reduce damaging oxidizing species within the cell. Once the redox balance is re-established, reducing agents like GSH or TRX might reduce GolDHase and restore its activity to allow the normal carbon flux and usage of Gol for carbon metabolism.

Materials and Methods

Plant material, bacterial strains, and reagents

Fruits from peach (*Prunus persica* cv. Flordaking) trees were harvested at Estación Experimental Agropecuaria San Pedro (INTA), Buenos Aires, Argentina, rapidly frozen with liquid N_2 and stored at –80 °C until use. *E. coli* TOP10 cells (Invitrogen, Carlsbad, USA) were used for cloning procedures and plasmid maintenance. Protein expression was carried out using *E. coli* BL21 Star (DE3) cells (Invitrogen). The substrates used to determine enzyme activity were from Sigma (Saint Louis, USA). All other reagents were of the highest purity available.

Phylogenetic analysis

We initially obtained 15 and 5 sequences coding for GolDHases in apple (*Malus* x *domestica*

Borkh.) (Velasco et al. 2010) and peach (*Prunus persica*) (Verde et al. 2013), respectively, from the Phytozome v9.1 server (http://www.phytozome.org/). After a detailed analysis, we discarded 3 sequences from apple and 1 sequence from peach due to the presence of large insertions and/or deletions. In addition, we downloaded 27 putative plant GolDHase sequences from the NCBI database (http://www.ncbi.nlm.nih.gov/). Sequences were analyzed with the program BioEdit 7.0.5.3 (Hall 1999) and aligned in the ClustalW server (http://www.genome.jp/tools/clustalw/). Tree reconstruction was performed with Seaview 4.4 (Gouy et al. 2010) using the Neighbor-Joining method and a bootstrap of 1000. The tree was prepared in the FigTree 1.4 program (http://tree.bio.ed.ac.uk/).

Molecular cloning of the genes coding for Ppe*GolDHase and* Ppe*TRXh*

Total RNA was extracted from fruit mesocarp according to Meisel et al. (2005). The RNA integrity was verified by agarose gel electrophoresis. First strand cDNA was synthesized using 0.25 µg of RNA, 200 pmol of oligo(dT) primer and 200 U of M-MLV reverse transcriptase (USB, Cleveland, USA). To amplify the gene coding for *Ppe*GolDHase we designed the specific oligonucleotides *Ppe*GolDHase-fo (CATATGGGCAAGGGAGGGATGTCTTC, *Nde*I site is underlined) and *Ppe*GolDHase-re (GTCGACCAAATTAAACATGACTTTTATGGC, *Sal*I site is underlined) based on the sequence reported by Yamada et al. (2001). The gene encoding *Ppe*TRX*h* was amplified using the specific oligonucleotides *Ppe*TRX*h*-fo (GCATATGGCGGAGGAAAATCAAGTC, *Nde*I site is underlined) and *Ppe*TRX*h*-re (CTCGAGAGCAGATGCAGTAGCAGT, *Xho*I site is underlined) based on the sequence published by Callahan et al. (1993).

Amplifications by PCR were performed with 1 µl of cDNA solution, 100 pmol of each primer and 2.5 U of *Taq* DNA polymerase (Fermentas, Glen Burnie, USA), with the following conditions: 5 min at 95 °C, 30 cycles of 1 min at 95 °C, 30 s at 50 °C and 1 min 30 s at 72 °C, followed by 10 min at 72 °C. DNA fragments were cloned into the pGEM-T Easy vector (Promega, Madison, USA) and the resulting constructions were used to transform *E. coli* TOP10 cells. The sequences of the amplified genes were confirmed by automated sequencing of two different clones (Macrogen, Seoul, Korea). The *Ppe*GolDHase

gene was subcloned into the pET19b vector (Novagen, Madison, USA) between *Nde*I and *Xho*I sites to obtain the recombinant protein linked to a His-tag at the N-terminus. The *Ppe*TRX*h* gene was subcloned into the pET24a vector (Novagen) between *Nde*I and *Xho*I sites to obtain the recombinant protein linked to a His-tag at the C-term.

Protein expression and purification

The construct [pET19b/*Ppe*GolDHase] was used to transform *E. coli* BL21 Star (DE3) cells. Expression of *Ppe*GolDHase was achieved by inoculating 1 l of LB media (supplemented with 100 µg/ml ampicillin and 0.1 mM $ZnCl_2$) with a 1/100 dilution of an overnight culture. Cells were grown at 37 °C and 200 rpm in an orbital shaker until OD_{600} reached ~0.6, induced with 0.2 mM IPTG at 25 °C overnight, harvested at 5000 x *g* at 4 °C for 15 min and kept at -20 °C until use. We also transformed *E. coli* BL21 Star (DE3) cells with the pET32a vector (Novagen) and the construct [pET24a/*Ppe*TRX*h*] for expressing *Eco*TRX and *Ppe*TRX*h*, respectively. Cells were grown in 1 l of LB media supplemented with 100 µg/ml ampicillin (for *Eco*TRX) and 50 µg/ml kanamycin (for *Ppe*TRX*h*) and expression of both enzymes was performed as described for *Ppe*GolDHase (see above).

All the enzymes were purified following the same procedure. The cell paste was resuspended with ~20 ml of *Buffer H* [25 mM Tris-HCl pH 8.0, 300 mM NaCl, 5% (v/v) glycerol, 10 mM imidazole] and disrupted by sonication. The resulting suspension was centrifuged twice at 35000 x *g* at 4 °C for 15 min and the supernatant was loaded in a 1 ml HisTrap column connected to an ÄKTA Explorer 100 purification system (GE Healthcare) previously equilibrated with *Buffer H*. After sample loading, the column was washed with 10 ml of *Buffer H* and the recombinant protein was eluted with a linear gradient of imidazole (10 to 300 mM, 50 ml). Fractions of 2 ml were collected and those containing the enzyme of interest were pooled and concentrated. Both *Eco*TRX and *Ppe*TRX*h* were incubated with 0.1 mM DTT for 10 min at room temperature to ensure complete reduction. The proteins were aliquoted, stored at -80 °C, and thawed only once just before use. Under these conditions, the proteins were stable and remained reduced for at least 3 months.

Protein methods

Protein electrophoresis was carried out under denaturing conditions (SDS-PAGE) as described by Laemmli (1970). The purity of the enzyme was evaluated by densitometry using the LabImage 2.7 program (Kapelan). Protein concentration was determined following the procedure described by Bradford (1976) using BSA as standard.

Native molecular mass determination

The native molecular mass of *Ppe*GolDHase was determined by using a Superdex 200 5/200 Tricorn column (GE Healthcare) equilibrated with *Buffer G* (50 mM HEPES pH 8.0, 100 mM NaCl). A calibration curve was constructed by plotting the K_{av} values versus $\log_{10}$ of the molecular mass from standard proteins (ovalbumin, 43 kDa; conalbumin, 75 kDa; aldolase, 158 kDa; ferritin, 440 kDa; and thyroglobulin, 669 kDa). The K_{av} was calculated as $(V_e-V_0)/(V_t-V_0)$, where V_e is the elution volume of the protein, V_0 is the elution volume of Dextran Blue and V_t is the total volume of the column.

Enzyme activity assay and determination of kinetic constants

The activity of *Ppe*GolDHase was determined by following changes in absorbance at 340 nm as described by Ohta et al. (2005), with minor modifications. In the direction of Gol oxidation, the standard assay mixture contained 100 mM Tris-HCl pH 9.0, 5 mM NAD^+, 400 mM Gol, and the proper amount of enzyme. In the direction of Fru reduction, the standard medium composition was 100 mM MOPS-NaOH pH 6.5, 0.2 mM NADH, 1 M Fru and enzyme in an appropriate dilution. Both reactions were carried out in a final volume of 50 µl at 25 °C in a 384-microplate reader (Multiskan Ascent, Thermo Electron Corporation, Waltham, USA). One unit of enzyme activity (U) is defined as the amount of enzyme catalyzing the reduction/oxidation of 1 µmol NAD^+/NADH in 1 min under the specified assay conditions.

Data of initial velocity (v) were plotted versus substrate concentration and kinetic constants were

calculated by fitting to the Hill equation: $v = V_{max}\ S^{nH} / (S_{0.5}{}^{nH} + S^{nH})$, where $S_{0.5}$ is the concentration of substrate (S) producing 50% of the maximal velocity (V_{max}), and n_H is the Hill coefficient. Kinetic constants for inhibitors were determined by using a modified Hill equation: $v = v_0 - (v_0 - V_{min})\ I^{nH} / (I_{0.5}{}^{nH} + I^{nH})$, where v_0 is the activity in absence of inhibitor, and $I_{0.5}$ is the concentration of inhibitor (I) producing 50% of the minimal activity (V_{min}). Fitting was performed by a nonlinear least-squares algorithm provided by the computer program Origin 7.0 (OriginLab Corporation). Kinetic constants are the mean of at least two independent sets of data, reproducible within ± 10%.

The activity of *Ppe*TRX*h* was determined as described by Holmgren (1977), following human insulin reduction at 630 nm. The standard assay mixture contained 100 mM potassium phosphate pH 7.0, 1 mM EDTA, 0.5 mM dithiothreitol, 0.13 mM human insulin, and different TRX concentrations.

Redox assays

Recombinant *Ppe*GolDHase (1 µM) was incubated in the presence of different agents at 25 °C with 25 mM Tris-HCl pH 8.0 and 10% (v/v) glycerol. Prior to the reducing treatment, the enzyme was ultrafiltrated by centrifugation at 10000 x *g* and 4 °C using a Vivaspin 500 device (Sartorius, Aubagne, France) to remove the oxidant. The GSH stock solution (100 mM) was freshly prepared in 0.5 M MOPS-NaOH pH 8.0 to avoid acidification and air oxidation. Reduction of *Ppe*GolDHase with *Eco*TRX and *Ppe*GolDHase was performed without DTT because it is a very efficient chelating agent for a range of biologically relevant metal ions, including Zn^{2+} (Krezel et al. 2001). Aliquots of the reaction mixtures were taken at regular time intervals and assayed for GolDHase activity. To analyze the oxidation and alkylating kinetics of *Ppe*GolDHase, we utilized the model of irreversible inhibition of enzymes described by Kitz and Wilson (1962). For detailed description of model see Appendix S1.

Determination of the midpoint reduction potential

The E_m of a protein is defined as the reduction potential in which the concentrations of oxidized and reduced proteins are equal (Schafer and Buettner 2001). The E_m of *Ppe*GolDHase and *Ppe*TRX*h*

were determined by redox titration with 2ME-HEDS, the reduced and oxidized species of β-mercaptoethanol, respectively. Two molecules of 2ME are formed from one molecule of HEDS, according to the reaction: HEDS ⇆ 2 H^+ + 2 e^- + 2 2ME. Different reduction potential values were obtained varying the relative concentrations of both species, maintaining the total concentration fixed at 10 mM, with the addition of 25 mM Tris-HCl pH 8.0. The E were calculated by using the Nernst equation applied to the reduction reaction stated above: $E = E_{m,8.0} - RT/nF\ ln\ ([2ME]^2/[HEDS])$, where $E_{m,8.0}$ is the E_m for 2ME-HEDS at pH 8.0 (-0,319 V) (Lees and Whitesides 1993), R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T is the temperature (298 K), n is the number of moles of electrons transferred in the reaction (2), and F is the Faraday constant (96485 J mol^{-1} V^{-1}) (Rouhier et al. 2004).

*Ppe*GolDHase (0.2 μM) and *Ppe*TRX*h* (5 μM) were incubated 1 h at room temperature in different redox buffers (redox equilibrium was reached, data not shown). To obtain the $E_{m,8.0}$ of *Ppe*GolDHase, aliquots of treated samples were taken and enzyme activity was assayed in the direction of Gol oxidation under standard conditions (see above). The $E_{m,8.0}$ of *Ppe*TRX*h* was determined by measuring the intrinsic fluorescence of the treated samples using an LS 55 Fluorescence Spectrometer (PerkinElmer, Llantrisant, UK). Excitation and emission spectra were obtained to determine optimal wavelengths for further measurements (data not shown). Fluorescence determinations were done at 280 nm (excitation) and 350 nm (emission) with a 5 nm slit, and data were collected for 5 s. Data of *Ppe*GolDHase activity and *Ppe*TRX*h* intrinsic fluorescence were plotted versus E and fitted to the Nernst equation using the program Origin 7.0 to calculate the E_m.

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Table 1. Kinetic parameters for the substrates of *Ppe*GolDHase. Reactions in the direction of Gol oxidation were done at pH 9.0, whereas those in the direction of Fru reduction were performed at pH 6.5, as described under “Materials and Methods”. Constants were calculated by fitting experimental data to the Hill equation. For calculating the catalytic efficiency, we used the theoretical molecular mass of the His-tagged *Ppe*GolDHase (41.9 kDa). Calculated parameters are the mean of at least two independent data sets ± the standard error.

Reaction	Substrate	$S_{0.5}$ (mM)	n_H	V_{max} (U mg^{-1})	Catalytic efficiency (M^{-1} s^{-1})
Gol oxidation	Gol	43 ± 5	1.5 ± 0.2	16.4 ± 0.8	265
	NAD^+	1.4 ± 0.3	0.8 ± 0.1		
Fru reduction	Fru	142 ± 6	1.2 ± 0.1	11.8 ± 0.2	56
	NADH	0.10 ± 0.04	0.9 ± 0.2		

Table 2. Inactivation of *Ppe*GolDHase by divalent cations. Enzyme activity was assayed in the direction of Gol oxidation in the presence of increasing concentrations of divalent cations. Experimental data were fitted to a modified Hill equation (for details, see “Materials and Methods”). Calculated parameters are the mean of at least two independent data sets ± the standard error.

Metal	$I_{0.5}$ (μM)
Cu^{2+}	0.031 ± 0.003
Hg^{2+}	0.097 ± 0.006
Ni^{2+}	16 ± 1
Zn^{2+}	40 ± 1
Co^{2+}	151 ± 9

Table 3. Effect of different agents on the activity of *Ppe*GolDHase. Second order constants were determined from secondary plots of k_{obs} versus effector concentration (for details see Figure S2), as described under "Materials and Methods". Calculated parameters are the mean of at least two independent data sets ± the standard error.

Effector	k'' (M^{-1} s^{-1})
Diamide[a]	12.3 ± 0.7
H_2O_2[b]	6.9 ± 0.1
SNP[a]	0.25 ± 0.02
GSSG[a]	0.035 ± 0.001
IAA[b]	17.70 ± 0.01

[a]Linear response

[b]Hyperbolic response

Legends to figures

Figure 1. Phylogenetic tree of plant GolDHases. The tree has two major branches containing sequences from mono and dicotyledonous plants. All the sequences from apple (*M. domestica*) are in the same branch. The sequence of peach fruit GolDHase is grouped with those from other *Prunus* species cloned from fruits. The sequences were number-coded for clarity (see Table S1 for more details). Node numbers represent the bootstrap values obtained during tree reconstruction, as described under "Materials and Methods".

Figure 2. Media optimization for expressing *Ppe*GolDHase. Different supplemented media were used for growing transformed cells to express the recombinant protein over night with 0.2 mM IPTG at 25 °C and 200 rpm. Cell pastes were resuspended in *Buffer H*, disrupted by sonication and, after centrifugation, crude extracts were assayed for enzyme activity in the direction of Gol oxidation, as described under "Materials and Methods". Data are the mean of two independent data sets ± the standard error.

Figure 3. Analysis of *Ppe*GolDHase expression and purification by SDS-PAGE. Lane 1, insoluble fraction; lane 2, soluble fraction (crude extract); lane 3, purified *Ppe*GolDHase; lane 4, molecular mass markers. The purity of the enzyme in lane 3 was higher than 95%, calculated by densitometry as described under "Materials and Methods".

Figure 4. Analysis of *Ppe*GolDHase activity at different pH values. Enzyme activity was measured in the direction of Gol oxidation (filled symbols) and Fru reduction (open symbols) at the stated pH values. Buffers used were Bis-Tris Propane (squares, pH 6.1 to 10.1), CAPS-NaOH (circles, pH 9.5 to 11.0), and MES-NaOH (up-triangles, pH 5.0 to 6.8). Data are the mean of two independent data sets ± the standard error.

Figure 5. Recovery of oxidized *Ppe*GolDHase activity by reduction with biological agents. The enzyme was oxidized until the activity was 0.05% of the initial value (white bar), desalted by ultrafiltration, and reduced with 10 mM GSH (dark gray bars) or 50 µM *Eco*TRX (light gray bars). Redox reactions were performed at 25 ºC and aliquots were taken at regular time intervals to measure enzyme activity in the direction of Gol oxidation, as described under "Materials and Methods". Enzyme activity data were related

to the value obtained at the beginning of each assay (black bar). Data are the mean of two independent data sets ± the standard error.

Figure 6. Determination of the E_m for *Ppe*GolDHase and *Ppe*TRX*h* by redox titration. Redox buffers (containing different [2ME]/[HEDS] ratios) were prepared using the Nernst equation, as described under "Materials and Methods". Proteins were incubated at 25 ºC with different buffers until redox equilibrium was reached (approximately 1 h). After that, *Ppe*GolDHase activity (in the direction of Gol oxidation under standard conditions) or *Ppe*TRX*h* intrinsic fluorescence were measured. Data of *Ppe*GolDHase activity (■) or *Ppe*TRX*h* intrinsic fluorescence (●) were related to the maximum value obtained within each assay and plotted versus the reduction potential. Data are the mean of two independent data sets ± the standard error.

Figure 7. Reduction of *Ppe*GolDHase by *Ppe*TRX*h*. The recombinant enzyme was oxidized with 0.2 mM H_2O_2 for 5 (●) or 10 (▲) min at room temperature and then incubated with increasing concentrations of *Ppe*TRX*h* for 30 min. A non-treated sample was used as control (■). Then, activity was measured in the direction of Gol oxidation, as described under "Materials and Methods". Enzyme activity data were related to the value obtained at the beginning of each assay. Data are the mean of two independent data sets ± the standard error.

Figure 8. Model for carbon partitioning in peach fruits. Gol imported from photosynthetic tissues is converted into Fru by GolDHase (reduced, yellow), with the concomitant production of NADH. Fru and NADH could be used for biosynthesis of other metabolites. Under conditions producing an oxidizing environment, GolDHase could be inactivated (oxidized, red) and Gol concentration might increase, which would protect cell integrity. When redox balance is restored, GolDHase could be reduced by reduced TRX or GSH, thus redirecting carbon toward other metabolic pathways. Abbreviations used are: ROS, reactive oxygen species; RNS, reactive nitrogen species. Membrane transporters (colored in green) are: GT, Gol transporter; ST, sucrose transporter; GPT, glucose-phosphate transporter. Numbers (in blue) stand for the following enzymes: 1, sucrose synthase; 2, hexokinase; 3, phosphoglucose isomerase; 4, UDP-Glc pyrophosphorylase; 5, phosphoglucomutase; 6, ADP-Glc pyrophosphorylase.

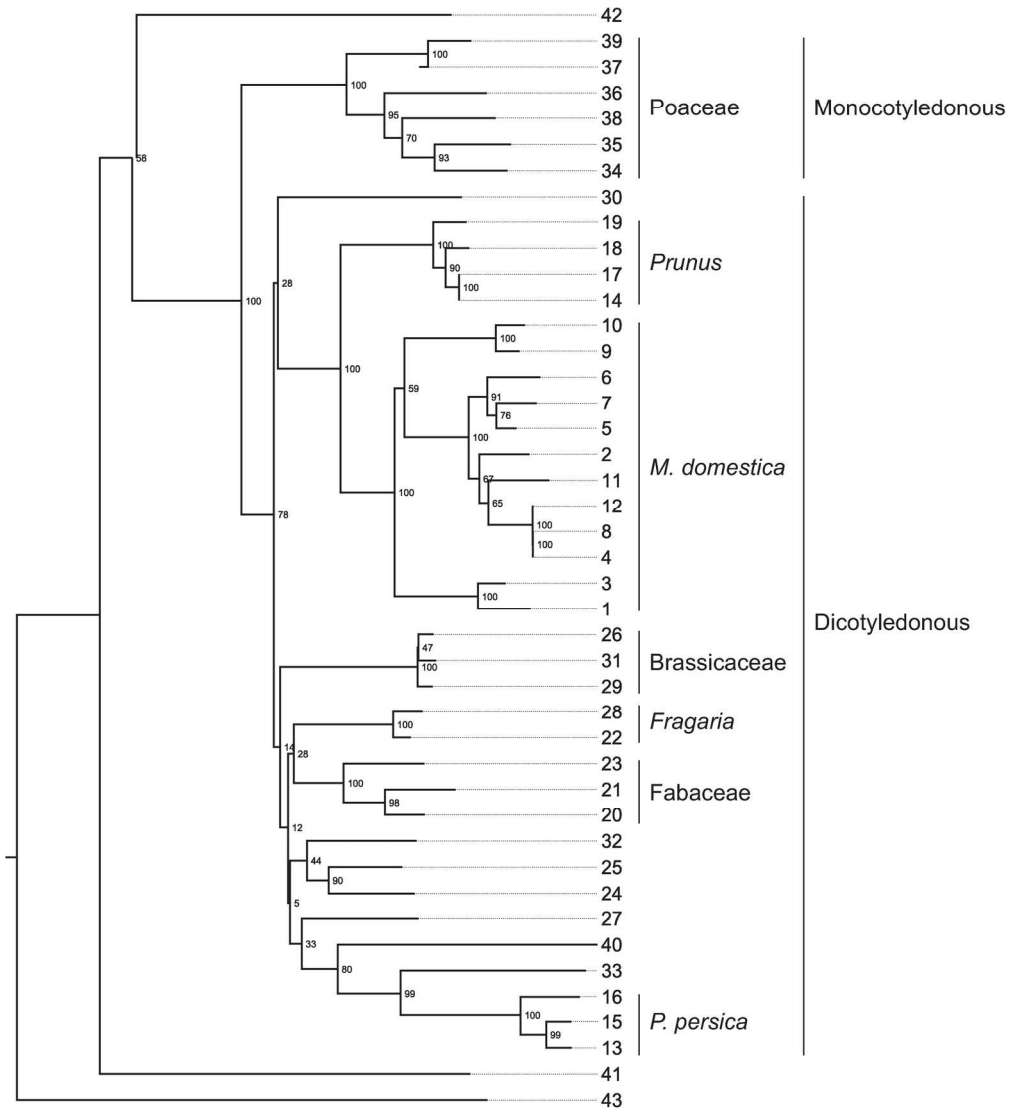

Figure 1. Phylogenetic tree of plant GolDHases. The tree has two major branches containing sequences from mono and dicotyledonous plants. All the sequences from apple (*M. domestica*) are in the same branch. The sequence of peach fruit GolDHase is grouped with those from other *Prunus* species cloned from fruits. The sequences were number-coded for clarity (see Table S1 for more details). Node numbers represent the bootstrap values obtained during tree reconstruction, as described under "Materials and Methods".
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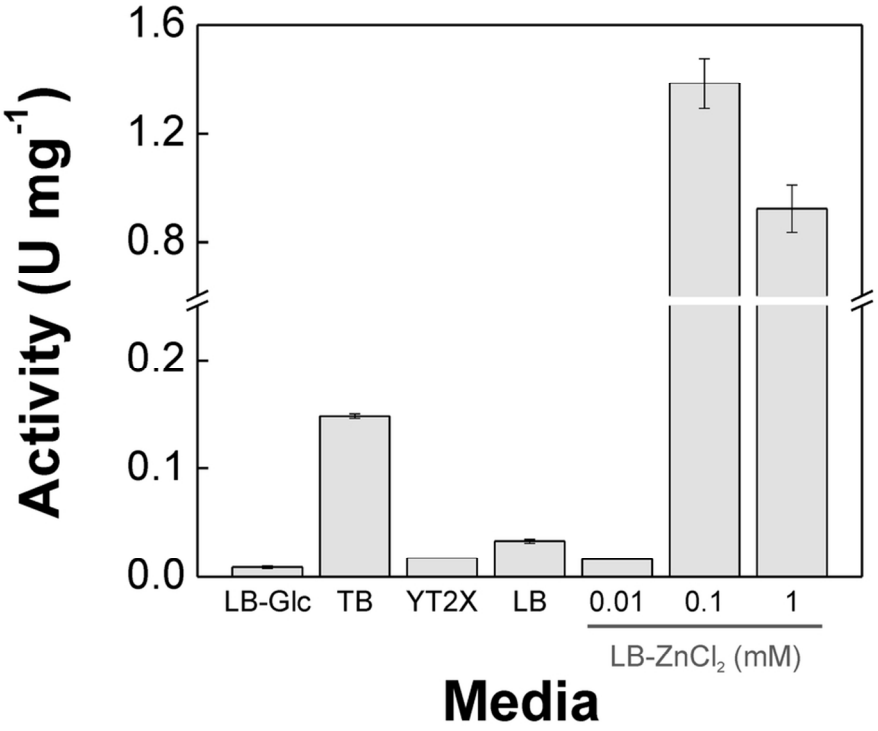

Figure 2. Media optimization for expressing *Ppe*GolDHase. Different supplemented media were used for growing transformed cells to express the recombinant protein over night with 0.2 mM IPTG at 25 °C and 200 rpm. Cell pastes were resuspended in *Buffer H*, disrupted by sonication and, after centrifugation, crude extracts were assayed for enzyme activity in the direction of GoI oxidation, as described under "Materials and Methods". Data are the mean of two independent data sets ± the standard error.
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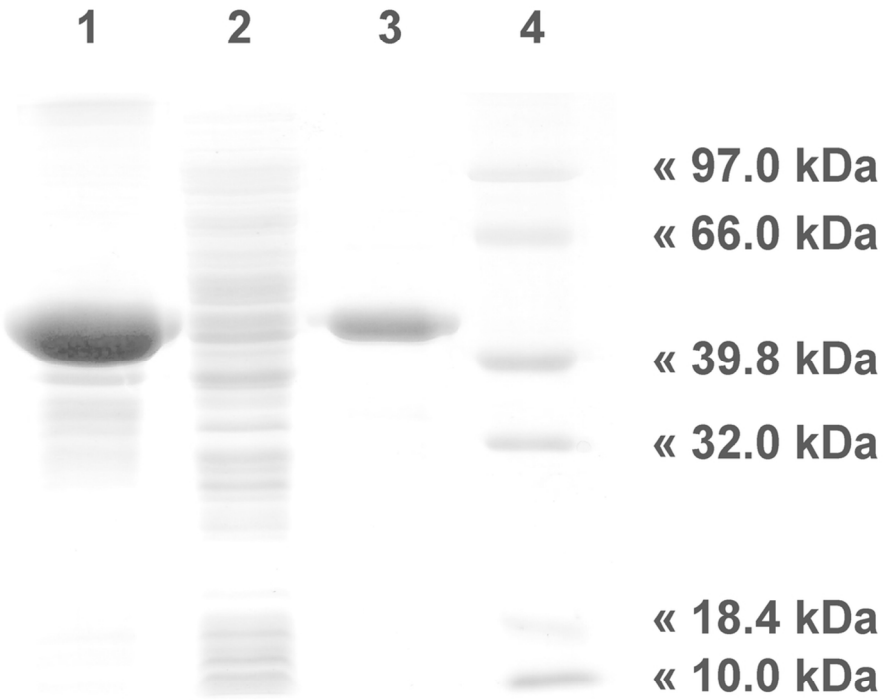

Figure 3. Analysis of *Ppe*GolDHase expression and purification by SDS-PAGE. Lane 1, insoluble fraction; lane 2, soluble fraction (crude extract); lane 3, purified *Ppe*GolDHase; lane 4, molecular mass markers. The purity of the enzyme in lane 3 was higher than 95%, calculated by densitometry as described under "Materials and Methods".
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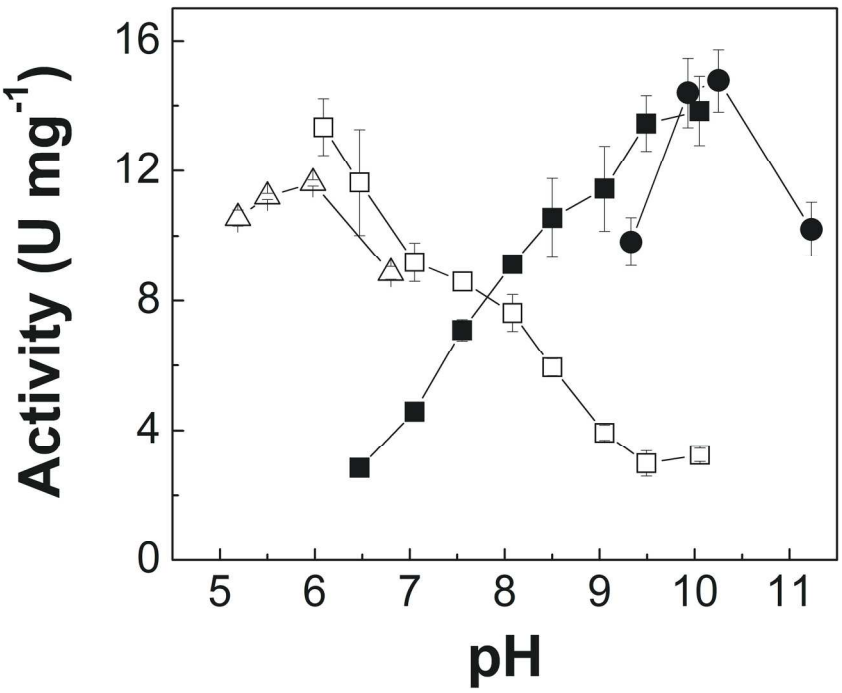

Figure 4. Analysis of *Ppe*GolDHase activity at different pH values. Enzyme activity was measured in the direction of Gol oxidation (filled symbols) and Fru reduction (open symbols) at the stated pH values. Buffers used were Bis-Tris Propane (squares, pH 6.1 to 10.1), CAPS NaOH (circles, pH 9.5 to 11.0), and MES-NaOH (up-triangles, pH 5.0 to 6.8). Data are the mean of two independent data sets ± the standard error.
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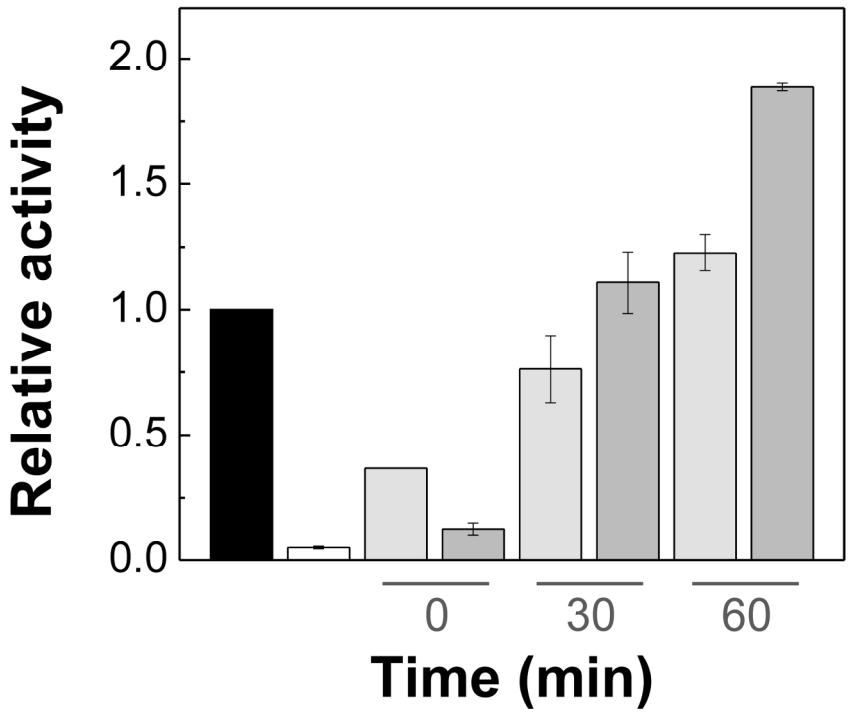

Figure 5. Recovery of oxidized *Ppe*GolDHase activity by reduction with biological agents. The enzyme was oxidized until the activity was 0.05% of the initial value (white bar), desalted by ultrafiltration, and reduced with 10 mM GSH (dark gray bars) or 50 µM *Eco*TRX (light gray bars). Redox reactions were performed at 25 ºC and aliquots were taken at regular time intervals to measure enzyme activity in the direction of Gol oxidation, as described under "Materials and Methods". Enzyme activity data were related to the value obtained at the beginning of each assay (black bar). Data are the mean of two independent data sets ± the standard error.
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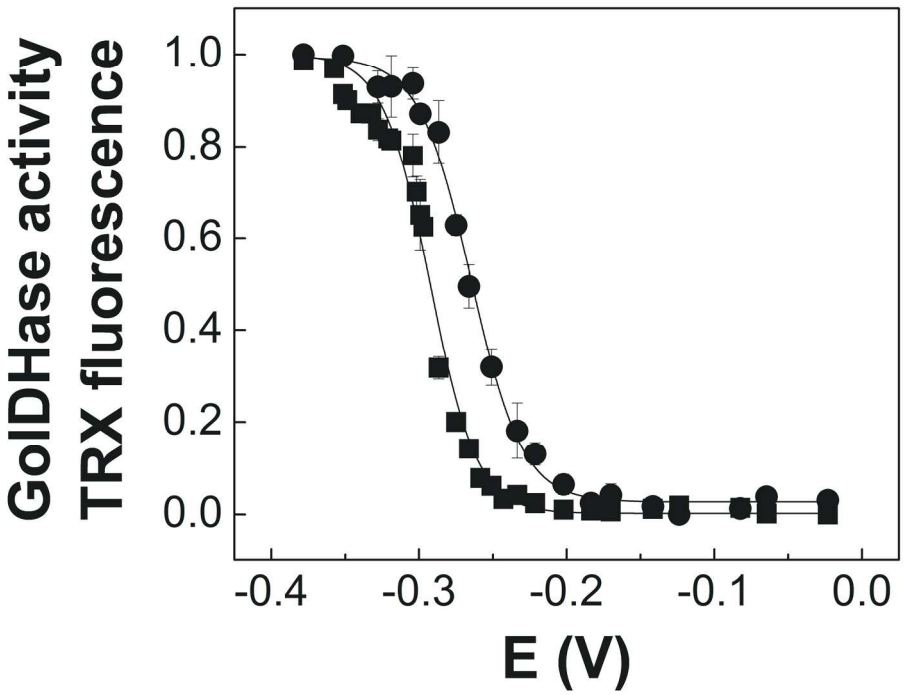

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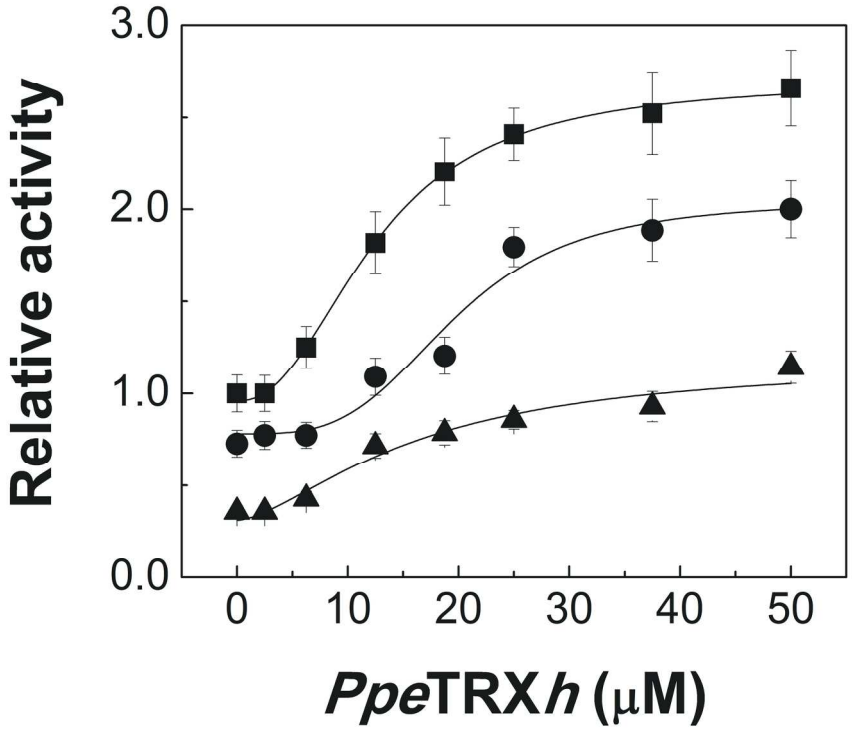

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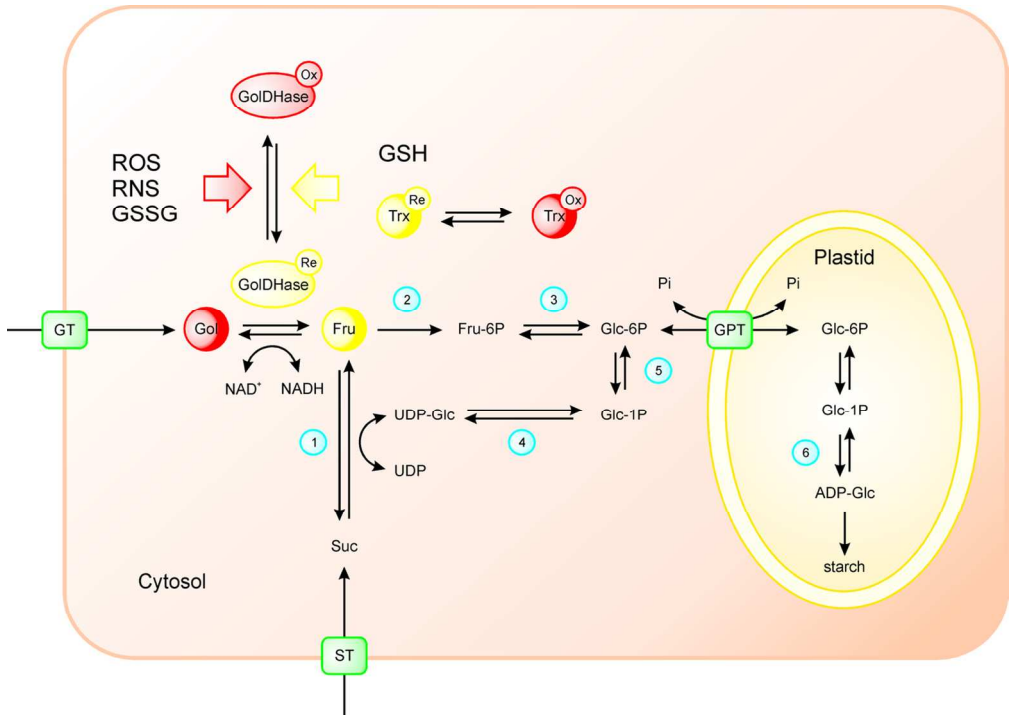

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120x85mm (300 x 300 DPI)